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(71) Applicant: THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Center for Technology Transfer, Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US).

(72) Inventor: PATERSON, Yvonne; 514 South 46th Street, Philadelphia, PA 19143 (US).

(74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, Woodland Falls Corporate Park, Suite 201, 210 Lake Drive East, Cherry Hill, NJ 08002 (US).

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(54) Title: SPECIFIC IMMUNOTHERAPY OF CANCER USING A LIVE RECOMBINANT BACTERIAL VACCINE VECTOR

(57) Abstract

Vaccines containing recombinant *Listeria monocytogenes* capable of expressing tumor specific antigens are provided. These vaccines, when administered to a host, are capable of inducing an immune response to the tumor specific antigen. Methods of using these vaccines in the suppression tumor formation are also provided.

SPECIFIC IMMUNOTHERAPY OF CANCER USING
A LIVE RECOMBINANT BACTERIAL VACCINE VECTOR

Introduction

The invention was made in the course of work supported
by the National Cancer Institute. The Government may have
certain rights in this invention.

Background of the Invention

Stimulation of an immune response is dependent upon the
presence of antigens recognized as foreign by the host immune
system. The discovery of the existence of tumor-specific
antigens has now raised the possibility of using a host's
immune system to intervene in tumor growth. Various mechanisms
of harnessing both the humoral and cellular arms of the immune
system are currently being explored for cancer immunotherapy.

Elements of the cellular immune response are capable of
specifically recognizing and destroying tumor cells. The
isolation of cytotoxic T cells (CTC) from tumor-infiltrating
cell populations or from peripheral blood suggests that such
cells play an important role in natural immune defenses against
cancer (Cheever et al., *Annals N.Y. Acad. Sci.* 1993 690:101-
112). CD+8 T cells (TCD8+) in particular, which recognize
Class I molecules of the major histocompatibility complex
(MHC)-bearing peptides of 8 to 10 residues derived from
proteins located in the cytosols, are believed to play an
important role in this response. There are now numerous
examples of both mouse and human TCD8+ that specifically
recognize tumor cells and have therapeutic activity after

adoptive transfer, in some cases inducing complete remission.
However, despite the potential for T cells to eradicate tumors,
it is obvious from the progressive growth of most cancers that
many tumors escape recognition by TCD8+ *in vivo*. The induction
of sufficient T cells *in vivo* has not been very effective.
Though a variety of tumors have been found to be immunogenic,
stimulation of an effective antitumor immune response has not
been found.

One explanation for this phenomena is that tumors may be
capable of delivering antigen-specific signals to T cells, but
not the costimulatory signals necessary for full activation of
T cells. Costimulation of T cells occurs when a surface
molecule, B7, on the presenting cells interacts with a T cell
molecule known as CD28. It has been observed that T cells
receiving the antigen-specific signal (but not B7) become
unresponsive. Many tumor cells do not carry the B7 protein,
therefore B7 has been added to cancer cells (Travis, J.,
Science 1993 259, 310-311). It has been demonstrated that
expression of the costimulatory ligand B7 on melanoma cells
induced the rejection of a murine melanoma *in vivo* (Townsend,
S.E. and Allison, J.P., *Science* 1993, 259, 368-370). This
rejection was found to be mediated by CD8+ T cells; CD4+ T
cells were not required. These results suggest that B7
expression may render tumor cells capable of effective antigen
presentation, resulting in their eradication *in vivo*.

The effects of localized secretion of cytokines on tumor
progression has also been studied. Secretions of low levels of
interleukin-2 (IL-2) in a mouse fibrosarcoma cell line
transfected with the human IL-2 gene introduced via a
retroviral vector was found to abrogate the tumorigenicity of
these cells and induce a long lasting protective immune
response against a subsequent challenge with a tumorigenic dose
of parent cells (Gansbacher et al., *J. Exp. Med.* 1990, 172,
1217-1224). In another study, cells from a spontaneously
arising murine renal cell tumor were engineered to secrete
large doses of interleukin-4 (IL-4) locally (Golumbek et al.,
Science 1991, 254, 713-716). Animals injected with the tumor

cells rejected the IL-4-transfected tumors in a predominantly
T cell-independent manner. However, these animals developed a
T cell-dependent systemic immunity to the parental tumor. The
systemic immunity was tumor-specific and mediated by CD8+ T
5 cells. These experiments suggest that it may be possible to
cure parental tumors by generating a systemic immune response
by the injection of genetically engineered tumor cells.

There is also evidence to suggest that some tumor cells
express low levels of class I molecules in vivo and in vitro.
10 Intracellular antigens must be processed before presentation to
CD+8 T cells by major histocompatibility complex (MHC) class I
molecules. The antigen processing efficiency of 26 different
human tumor lines has been studied (Restifo et al., J. of Exp.
Med. 1993, 177, 265-272). Three different cell lines, all
15 human small cell lung carcinoma, consistently failed to process
endogenously synthesized proteins for presentation to the T
cells. Pulse chase experiments showed that MHC class I
molecules were not transported by these cells lines from the
endoplasmic reticulum to the cell surface. Northern blot
20 analysis showed that these cells contained little or no mRNA
encoding MHC-encoded proteosomes and transporter genes.
Treatment with interferon γ enhanced expression of these mRNAs
and reversed the observed functional and biochemical deficits.
Thus, potential therapeutic applications which include
25 enhancing antigen processing at the level of transcription of
MHC-encoded proteosome and transporter genes was suggested.

Immunizing patients with recombinant BCG (bacille
Calmette-Guèrin) or Salmonella bacteria carrying a gene coding
for an antigenic peptide has also been suggested as an oral
30 tumor immunotherapy (Boon et al. Annu. Rev. Immunol. 1994, 12,
337-65). Orally administered live attenuated Salmonella
recombinant vaccine, which expressed the full length P. berghei
circumsporozite antigen, has been shown to protect mice against
malaria. This immune response was mediated by the induction of
35 CD8+ T cells (Aggarwal et al., J. of Exp. Med. 1990, 172, 1083-
1090). It is suggested that live attenuated Salmonella
recombinants may be useful in the study of other diseases where

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CTC-mediated immunity may be important, however, no other
experiments were reported. BCG has also been implicated as a
novel live-vaccine vehicle which may prove useful in
stimulating both humoral and cellular immune response to a wide
5 variety of viral, bacterial and protozoal antigens (Stover et
al., Nature 1991, 351, 456-460).

It has now been found that the immune response to a
tumor-specific antigen can be induced by the administration of
a vaccine vector comprising a recombinant form of the
10 intracellular bacterium Listeria monocytogenes which express a
tumor specific antigen or fragment thereof. This vaccine
vector has been found to be useful in decreasing the size of
existing tumors and in inhibiting formation of primary tumors.
No other stimulation following antigen presentation was
15 required to induce this response.

Summary of the Invention

An object of the present invention is to provide a method
of inducing an immune response to a tumor-specific antigen in
a host having cancer which comprises administering to the host
20 an effective amount of a vaccine comprising a recombinant form
of Listeria monocytogenes capable of expressing a tumor
specific antigen or fragment thereof.

Another object of the present invention is to provide a
use for a vaccine comprising a recombinant form of Listeria
25 monocytogenes capable of expressing a tumor specific antigen or
fragment thereof to induce an immune response to a tumor
specific antigen.

Another object of the present invention is to provide
vaccines for the treatment of cancer which comprise a
30 recombinant form of Listeria monocytogenes capable of
expressing a tumor specific antigen or fragment thereof.

Another object of the present invention is to provide a
method of inhibiting the formation of tumors in a host which
comprises administering to the host an effective amount of a
35 vaccine comprising a recombinant form of Listeria monocytogenes

capable of expressing a tumor specific antigen or fragment
thereof.

Brief Description of the Drawings

Figures 1 through 4 provide line graphs from experiments
5   wherein mice were immunized with either saline (●), L.
monocytogenes (■), or recombinant L. monocytogenes transformed
to express influenza nucleoprotein (LM-NP) (♦) and then
subsequently challenged with either CT26 or RENCA which had
been transfected with the same influenza nucleoprotein (NP)
10   gene that was used to transform the L. monocytogenes vector
(CT26-NP or RENCA-NP, respectively) or with the parental CT26
or RENCA line.

Figure 1 provides data from experiments wherein mice in
each immunization group were challenged with parental RENCA.
15   Figure 2 provides data from experiments wherein mice in
each immunization group were challenged with parental CT26.

Figure 3 provides data from experiments wherein mice from
each immunization group were challenged with RENCA transfected
with the same NP used to transform the L. monocytogenes (RENCA-
20   NP).

Figure 4 provides data from experiments wherein mice from
each immunization group were challenged with CT26 transfected
with the same NP used to transform the L. monocytogenes (CT26-
NP).

25   Figure 5 is a bar graph which provides data from
experiments wherein it was shown that CTL generated by
immunizing Balb/c mice with LM-NP can kill tumor cells CT26 and
RENCA that express NP in vitro. Figure 5A shows effectors
stimulated with A/PR/8. Figure 5B shows effectors stimulated
30   with peptides.

Figure 6 is a bar graph which provides data from
experiments wherein it was shown that immunization by LM-NP
causes elimination of RENCA-NP tumor growth. ●

Figure 7 is a bar graph which provides data from
35   experiments wherein it was shown that immunization by LM-NP
causes cessation of CT26-NP tumor growth.

Figure 8 is a bar graph which provides data from
experiments wherein it was shown that inhibition of tumor
growth is caused by CD8+ T cells.

Detailed Description of the Invention

5   The immune response to L. monocytogenes has been shown
to be a TH1, CD4⁺ T cell and CD8⁺ T cell response with only very
weak humoral responses being engendered. Recombinant forms of
the wild-type bacterium have been developed which express the
foreign proteins β-galactosidase (Schafer et al., J. Immunol.
10   1992, 149, 53-59), influenza nucleoprotein and HIV gag and nef
gene product. Recombinant techniques have been developed to
stably integrate these proteins into the Listerial chromosome
in such a way that they are secreted by the bacterium. All of
these recombinant vectors elicit strong, antigen specific, CTC
15   responses in vivo. Thus, this bacterium serves as an ideal
vaccine vector for boosting the CTC response to tumor specific
proteins and a unique system to prime the cellular immune
response as a vaccine against cancer.

Administration of a live vector such as L. monocytogenes
20   results in a long lasting cellular immunity which often cannot
be induced with killed preparations or soluble protein and
adjuvant.   A unique feature of the life-cycle of L.
monocytogenes is that it invades the host cell and is taken up
into a phagosome from which it escapes and then lives and
25   replicates in the cytoplasm. Tilney, L.G. and D.A. Portnoy, J.
Cell Biol. 1989 109, 1597. Thus, the L. monocytogenes vector
provides the ability to target foreign proteins and fragments
of proteins to the class I MHC restricted pathway. In addition
to being a more efficacious vector, L. monocytogenes, which is
30   a gram-positive organism, is also much safer than many other
live vectors since it is very susceptible to most antibiotics,
including penicillin.   It also does not have the problems
associated with toxicity from endotoxin which gram negative
vectors such as Salmonella sp. present. Pre-existing immunity
35   which could prevent effective boosting by a vector which has
already been widely used as a vaccine, e.g., Vaccinia or BCG,

is not likely to be a problem for *L. monocytogenes*, which has not been used previously in vaccine development. Mutant strains of *L. monocytogenes*, which are avirulent but still protective, are also available for testing as potential vaccine
5 candidates.

Using a model murine system, it has now been found that *L. monocytogenes* can induce an immune response against a protein expressed by tumor cells. This immune response causes the rejection of transferred tumor cells to healthy, immunized
10 mice and effects tumor growth in mice in which tumor growth has already been initiated. See Figures 3 through 7.

The ability of a vaccine comprising recombinant *L. monocytogenes* to convey specific protective immunity against the growth of CT26, a mouse colorectal carcinoma tumor and
15 RENCA, a murine renal carcinoma, was examined. In preliminary experiments, *L. monocytogenes* was engineered to secrete nucleoprotein (NP) from A/PR/8/34 as a fusion protein with a major secreted Listerial protein, listeriolysin O (LLO), the product of the hemolysin gene. LLO is normally expressed and
20 secreted in a host vacuole by *L. monocytogenes* and is required for escape of the bacteria into the cytoplasm. The ability of NP secreting *L. monocytogenes* recombinants to target the class I pathway of antigen processing for recognition by bulk influenza specific T cells from three strains of mice was
25 tested. It was determined that the LLO-NP fusion proteins are appropriately processed for presentation by the three MHC class I haplotypes to which the A/PR/8/34 response is restricted, i.e., K^d, D^b and K^k. Immunization of Balb/c mice with varying doses of LM-NP was demonstrated to result in a strong anti-NP
30 CTC response.

In further experiments, mice were divided into three groups. One group was immunized with one-tenth of an LD_{50} of wild-type *L. monocytogenes*, one group was immunized with sterile saline, and the third group was immunized with a
35 recombinant *L. monocytogenes* vaccine vector which was transformed to secrete influenza nucleoprotein (LM-NP). After two weeks, each group received a similar booster immunization.

This immunization schedule was determined to produce strong CTC responses against influenza nucleoprotein. Two weeks after the last immunization, animals in each group were challenged subcutaneously with a tumoricidal dose of either CT26 or RENCA
5 which had been transfected with the same influenza nucleoprotein gene that was used to transform the *L. monocytogenes* vector (CT26-NP or RENCA-NP, respectively) or with the parental CT26 or RENCA line. Tumor growth was monitored. As shown in Figure 3 and 4, animals which received
10 LM-NP as the vaccine and which were challenged with the relevant tumor cell expressing NP were protected from further tumor formation. In the CT26-NP group, after 25 days, 6 of the animals showed no detectable tumor growth, 3 had tumors of less than 5.0 mm and one had a tumor of 9.0 mm (see Figure 4). In
15 the RENCA-NP group, none of the animals showed any signs of tumor growth (see Figure 3). In contrast, all the mice in the other groups developed tumors between 1.5 and 3.0 cm (see Figure 1 and 2).

The ability of LM-NP to cause regression and depletion
20 of existing tumors was also demonstrated. Tumor cells (either CT26 or RENCA cells) were introduced subcutaneously into mice. After the formation of measurable tumors, the mice were divided into three separate groups. A first group of mice received LM-NP, a second group of mice received wild type *Listeria*
25 *monocytogenes* and a third group of mice received no further treatment. Mice in groups 1 and 2 were given a subsequent booster of either LM-NP or wild type *Listeria monocytogenes*, respectively. As shown in Figures 6 and 7 only the mice that received the LM-NP vaccine showed regression of tumor growth to
30 the point where the tumor was no longer visible.

Vaccines of the present invention require that a tumor specific antigen be known or identified for the cancer. A number of such antigens have been identified. They include the antigen *bcr/abl* in leukemia, HPVE6 and E7 in the oncogenic
35 virus associated with cervical cancer, MAGE1 and MZ2-E in melanoma, and MVC-1 in breast and pancreatic carcinoma. As will be obvious to those of skill in the art upon this

disclosure, however, the invention is applicable to any tumor
antigen.

For example, the chronic myeloid leukemia (CML) antigen
p210$^{ber-abl}$, which is expressed in 90 to 95% of CML patients, is
5 a tumor-specific antigen by virtue of its unique junctional
sequence.   L. monocytogenes recombinants which secrete
oligopeptides from the joining region of p210$^{ber-abl}$ are
constructed by any technique which permits the insertion of
foreign genes directly into the bacterial chromosome and allows
10 the secretion of the gene product using the LLO signal
sequence.   The recombinant L. monocytogenes can then be
administered as a vaccine, either alone or in the presence of
a pharmaceutically suitable carrier, to protect against CML
induced by retroviral expression of p210$^{ber-abl}$. One of skill in
15 the art upon this disclosure could routinely extend this
approach to other tumor antigens.

Very stable transformants which secrete a number of large
viral proteins have been produced routinely using techniques
routine to those of skill in the art. Several techniques for
20 producing recombinant L. monocytogenes are known.

For example, the integration in the Listerial chromosome
as a result of a transposon insertion is described by Sun et
al., Infection and Immunity 1990, 58, 3770-3778 in the
construction of DP-L967.   Transposon mutagenesis has the
25 advantage that a stable genomic insertion mutant can be formed
but the disadvantage that the position in the genome where the
foreign gene has been inserted is unknown.

Cloning of the gene into a prfA-containing vector and
using this plasmid to complement a prfA(-) Listeria mutant has
30 been used to construct DP-L2028. DP-L2028 is the influenza NP
expressing strain used in the tumor protection experiments.

Several approaches may be taken to express the tumor
antigen in Listeria sp. as will be understood by one skilled in
the art based upon this disclosure. One example is to generate
35 a fusion protein of the selected tumor antigen and a Listerial
protein such as Listeriolysin O or PI-PLC. Another way is
through the use of a signal sequence, for a secreted Listerial

protein such as hemolysin or phospholipases, employed
downstream of a Listerial promoter. The promoters of various
L. monocytogenes genes may be used to express foreign antigens.
In addition, these genes may be used to generate fusion
5 proteins with foreign antigens. For example, promoters for the
genes hly, actA, plcA, plcB and mpl, which encode the Listerial
proteins hemolysin, actA (a surface protein necessary for host
cell actin assembly and essential for cell to cell spread of
the bacterium), phosphotidylinositol-specific phospholipase,
10 phospholipase C, and metalloprotease, respectively, can be
used.

A more preferred method for producing these recombinants
is integration into the Listeria chromosome by homologous
recombination with a temperature sensitive plasmid.   This
15 method can be used to produce stable transformants that secrete
the protein of interest.   Unlike the case with transposon
mutagenesis, the site of insertion is known.   This method
allows for the routine insertion of any gene of interest into
the chromosome of L. monocytogenes which is then expressed
20 under the control of a L. monocytogenes promoter. One such
promoter, the hemolysin promoter, regulates the expression of
hly, the Listerial gene which encodes LLO, an abundantly
synthesized and secreted protein. It has been shown that the
inclusion of the LLO signal sequence allows for the secretion
25 of the expressed protein outside the bacterial cell wall. The
construction of these stable recombinants of L. monocytogenes
utilizes a region of its chromosome that can act as a site for
insertion without disrupting bacterial genes necessary for the
growth and spread of the organism (Camilli et al., Mol.
30 Microbiol. 1993, 8, 143-157).   This homology region is
introduced into the shuttle vector pKSV7, a temperature
sensitive plasmid that functions in both E. coli and L.
monocytogenes. An EcoR1 site near its center is then used to
insert a series of DNA fragments between the two halves of this
35 region.   After the addition of a polylinker, the promoter
sequence of the Listeria hemolysin gene is inserted. Along
with the promoter, downstream sequence information for the

first 26 amino acids of the LLO protein (the signal sequence)
and four additional amino acids is included to ensure proper
processing · of the signal sequence. The transcription
termination sequence of the hemolysin gene is also included to
5 ensure that stable and regulated synthesis of all transcripts
synthesized. These hemolysin regulatory sequences are used to
promote the abundant synthesis and secretion of any adjoining
downstream gene.

The vaccines of the present invention can be administered
10 to a host, either alone or in combination with a
pharmaceutically acceptable carrier, in an effective amount to
induce an immune response to a tumor-specific antigen. By
"host" it is meant to include any organism capable of
sustaining cancerous cells, preferably a human. By "effective
15 amount" it is meant a concentration of recombinant L.
monocytogenes capable of expressing a tumor specific antigen
which is capable of invoking an immune response in T cells
which will eradicate cells containing this antigen. Such
amounts can be routinely determined by one of skill in the art
20 upon this disclosure. By "pharmaceutically acceptable carrier"
it is meant to include, but is not limited to, sterile,
distilled water, saline, phosphate buffered solutions or
bicarbonate buffered solutions. The pharmaceutically
acceptable carrier selected and the amount of carrier used will
25 depend upon the mode of administration. Administration may be
oral, parenteral, intranasal, intramuscular, intravascular,
intrarectal, intraperitoneal, or any one of a variety of well-
known routes of administration. The route of administration
may be selected in accordance with different tumors. For
30 example, for treatment of cancers of the alimentary tract, oral
administration may be used. For treatment of colorectal
cancer, intra-rectal immunization may be used. For the
treatment of ovarian or pancreatic cancer, intraperitoneal
administration may be used. The vaccines of the present
35 invention may be administered in the form of elixirs, capsules
or suspensions for oral administration or in sterile liquids
for parenteral or intravascular administration. The vaccines

may be stored frozen, at 4°C, at room temperature or
lyophilized.

In a preferred embodiment, the vaccines of the present
invention are administered to a host either alone or in
5 combination with another cancer therapy to inhibit or suppress
the formation of tumors.

Thus, the vaccines of the present invention can be used
to protect people at high risk for cancer because of familial
genetics or other circumstances that predispose them to certain
10 types of cancer, e.g., cervical cancer in women whose husbands
have papilloma virus. In addition, the vaccines can used as a
cancer immunotherapy after debulking of tumor growth by
surgery, conventional chemotherapy or radiation treatment.
Following such treatments, recombinant L. monocytogenes
15 expressing the tumor antigen can be administered. The CTC
response to the tumor antigen produced by the vaccine will
destroy remaining metastases and prolong remission from the
cancer. It is also believed that the vaccines of the present
invention can be used to effect the growth of previously
20 established tumors.

The following examples are provided for illustrative
purposes only and are not intended to limit the invention.

EXAMPLES

Example 1

25   A sequence encoding the first 420 amino acids of
Listeriolysin O (LLO) and its promoter along with some upstream
regulatory sequences was PCR amplified from L. monocytogenes
chromosomal DNA (wild type strain 10403s) and ligated to PCR
amplified DNA encoding NP, derived from plasmid pAPR502.
30 (Young, J.F., U. Desselberger, P. Graves, P. Palese and A.
Shatzman, "Cloning and Expression of influenza virus genes",
The Origin of Pandemic Influenza Viruses, W.G. Laver, eds.,
Elsevier, New York, 1983, p. 129). The construction resulted
in an in-frame fusion plus the addition of two amino acids at
35 the site of the fusion junction. The fusion was cloned into
the shuttle plasmid pAM401, a shuttle vector able to replicate

in both gram+ and gram- bacteria which contains a gram+
chloramphenicol resistance gene and a gram- tetracycline
resistance gene (Wirth, R., F.Y. An and D.B. Clewell, *J.
Bacteriol.* 1986, 165, 831). The resultant plasmid, pDP1659,
was introduced into wild type *L. monocytogenes* (strain 10403s)
by electroporation to yield *L. monocytogenes* strain DP-L1659.
This recombinant strain was clearly able to make and secrete a
fusion protein of the predicted size (105 kD) as determined by
Western blot analysis of the secreted proteins in the culture
supernatants using anti-LLO polyclonal antiserum and anti-NP
monoclonal antibody. The presence of the fusion gene under the
control of the LLO promoter in a multicopy plasmid resulted in
reduced secretion of the chromosomally encoded LLO, but not to
the extent that it prevented escape of the bacteria from the
vacuole or subsequent intracytoplasmic growth. However, this
strain was not stable in the absence of chloramphenicol.

     To construct *L. monocytogenes* strain, DP-L2028, which is
stable *in vivo* and which was used in Examples 2 through 6,
plasmid pDP-1659 was modified by inserting the prfA gene from
10403s and then used to transform a prfA-*L. monocytogenes*
mutant DP-L1075. This resulted in *L. monocytogenes* strain DP-
L2028 which secretes the LLO-NP fusion protein stably *in vivo*
and *in vitro*.

Example 2: Treatment of Mice with LM-NP

     One hundred and twenty Balb/c mice were divided into
three groups of 40. One group was immunized with one-tenth of
an LD50 of wild-type *L. monocytogenes*, one group was immunized
with sterile saline and the third group was immunized with a
recombinant *L. monocytogenes* vaccine vector transformed to
secrete influenza nucleoprotein (LM-NP). After two weeks, each
group received a similar booster immunization. This
immunization schedule was determined to produce strong CTC
responses against influenza nucleoprotein. Two weeks after the
last immunization, animals in each group were challenged
subcutaneously with either CT26 or RENCA which had been
transfected with the same influenza nucleoprotein gene that was

used to transform the *L. monocytogenes* vector (CT26-NP or
RENCA-NP, respectively) or with the parental CT26 or RENCA
line. Each mouse was administered 5 x 10⁵ tumor cells, which
is 50 times the tumoricidal dose. Tumor growth was monitored
every two days in these six groups of animals. Results from
this study are shown in Figures 1 through 4. The only group
showing any protection from the tumoricidal dose was the
animals which received LM-NP as the vaccine and which were
challenged with the relevant tumor cell expressing NP. In the
CT26-NP group, after 25 days, 6 of the animals showed no
detectable tumor growth, 3 had tumors of less than 5.0 mm and
one had a tumor of 9.0 mm. In the RENCA-NP group, none of the
animals showed any signs of tumor growth. In contrast, all the
mice in the other groups have tumors between 1.5 and 3.0 cm.

     In order to maintain the foreign NP gene, CT26-NP is
usually maintained on the antibiotic G418. It is believed that
the small number of CT26-NP tumor cells that grew in the LM-NP
immunized mice are cells which have lost the NP gene in the
absence of G418.

**Example 3: CTL generated by immunizing Balb/c mice with LM-NP
             can kill tumor cells CT26 and RENCA that express NP
             in vitro**

     Mice were immunized with 0.1 LD₅₀ of LM-NP. Two weeks
later, the mice were sacrificed and primary cultures were set
up of spleen cells with either influenza infected (A/PR8/34)
splenocytes (Figure 5A) or with a synthetic peptide 147-158
known to represent the immunodominant epitope of the NP protein
(Figure 5B). After four days in culture, the cytolytic
activity of both populations was measured against CT26-NP,
RENCA-NP and the parental cell lines CT26 and RENCA. A
positive control was included (P815, a mastocytoma tumor cell
line known to be efficiently lysed by H-2ᵈ restricted CTL in
the presence of the peptide or when infected by A/PR8/34). As
Figure 5A shows, RENCA-NP and CT26-NP, but not the parental
lines, were lysed by NP specific effectors induced by
immunizing with LM-NP and expanded with A/PR8/34. In Figure

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5B, a similar experiment in which the effectors were expanded
with peptide show similar results.

**Example 4: Immunization by LM-NP will cause elimination of
RENCA tumor growth**

5   In this experiment, immunization with LM-NP after tumor
growth had been initiated caused regression and depletion of
tumors. Tumor cells (5 x 10⁵) were introduced subcutaneously
to 30 mice. On Day 13, after measurable tumors (5 mm) had
grown in the mice, they were divided into three groups of ten.

10   Ten mice received LM-NP, 10 mice received wild type *Listeria
monocytogenes* and ten received no further treatment. On Day 23
the mice were immunized again with either LM-NP or wild type
Listeria monocytogenes. As Figure 6 shows, only the mice that
received the LM-NP vaccine show regression of tumor growth to

15   the point where the tumor was no longer visible in 9 out of 10
mice.

**Example 5: Immunization by LM-NP will cause cessation of CT26-
NP tumor growth**

      The experiment described in Example 4 was also done with

20   the colorectal CT26-NP tumor cells. CT26-NP is a much faster
growing tumor and is also more unstable in its expression of
NP. Nevertheless, in this experiment, it was also found that
immunization by LM-NP after tumor growth has been initiated
halts tumor growth.   Tumor cells (5 x 10⁵) were introduced

25   subcutaneously to 30 mice. On Day 10, after measurable tumors
(5 mm) had grown in the mice, they were divided into three
groups of ten. Ten mice received LM-NP, 10 mice received wild
type *Listeria monocytogenes*, and 10 mice received no further
treatment. On Day 17 the mice were immunized again with either

30   LM-NP or wild type *Listeria monocytogenes*. As Figure 7 shows,
only the mice that received the LM-NP vaccine show a change in
tumor growth. However, unlike the case with RENCA, regression
of growth was not observed in as many mice.   This may be
because by Day 17, instability of the CT26-NP tumor cells

35   resulted in many of the tumor cells losing the NP antigen.

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**Example 6: Inhibition of tumor growth is caused by CD8+ T
cells**

      In this experiment, 30 mice were immunized with LM-NP
using the same protocol as discussed in Example 2. Ten days

5   after the last immunization, 10 mice were depleted of CD8+
cells by immunizing with antibody 2.43 (specific for the CD8
molecule); 10 mice were depleted of CD4+ cells by immunizing
with GK 1.5 (specific for the CD4 molecule); and 10 mice were
left with a complete T cell repertoire.   (The protocol for

10   depletion of CD8+ or CD4+ T cells was that as described by A.
Kruisbeek, *Current Protocols In Immunology*, Coligan et al.,
eds, John Wiley & Sons, Inc., 1994, V.1, 4.1.1-4.1.2). After
T cell depletion, the mice were challenged subcutaneously with
5 x 10⁵ CT26-NP cells per mouse. As a control, 10 naive mice

15   were also challenged with the same dose. As Figure 8 shows,
the group of mice in which the CD8+ T cell subset was depleted
showed similar tumor growth to the control (naive) group of
mice. The mice in which the CD4+ T cell subset was depleted
showed reduced protection against tumor growth, indicating that

20   CD4+ cells play an accessory response in the control of tumor
growth; and the mice with a complete T cell repertoire show
protection against tumor growth induced by the LM-NP vaccine.